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PRINCIPAL INVESTIGATOR: Angera H. Kuo

CONTRACTING ORGANIZATION: Georgetown University Medical Center
Washington, DC 20057

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6. AUTHOR(S)
Angera H. Kuo

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Georgetown University Medical Center
Washington, DC 20057

E-Mail: kuoa@georgetown. edu

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13. ABSTRACT (Maximum 200 Words)

Angiogenesis, formation of new blood vessels, plays a key role in breast cancer growth by providing a method for hematogenous spread of the tumor, as well as providing nutrients for tumor metastasis. Rational treatment strategies for breast cancer must take into account the molecular mechanisms by which cancer develops, maintain its growth and finally spread to other parts of the body. The pleiotrophin (PTN) signaling pathway is known to be important in angiogenesis and breast cancer growth, but the exact mechanism by which PTN acts has not been elucidated. Recently, we identified a cell-surface receptor tyrosine kinase, anaplastic lymphoma kinase (ALK) receptor, as a receptor for PTN. In this report, due to lack of a good ALK antibody, generation of a tagged full-length ALK was created to facilitate the detection of ALK. Utilization of a tagged ALK-ECD construct showed that it co-immunoprecipitated with PTN. Although the precise region of PTN binding site is still unknown (due to unsuccessful attempts at creating ALKΔ368-406), success at creating other ALK mutants showed that the site-directed mutagenesis could be employed to create ALK mutants.

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INTRODUTION

Angiogenesis plays key roles in breast cancer by providing essential nutrients for tumor growth, and by providing access to the general circulation for tumor metastasis (1). Signaling pathways employed by well known angiogenic factors, such as basic fibroblast growth factor (bFGF), have been well defined (2). The signaling pathway of pleiotrophin (PTN) has also been shown to be important in angiogenesis and breast cancer growth, although the exact mechanism by which PTN acts has not been elucidated. Recently, we identified a receptor tyrosine kinase, anaplastic lymphoma kinase (ALK), as a receptor for PTN (3). Here we report the PTN/ALK interaction in an *in vitro* assay.

BODY

The goal of Aim 3 was to determine the molecular interface of PTN with ALK. The first step towards this goal was to clone ALK cDNA into mammalian expression vector with epitope tag. ALK is a receptor tyrosine kinase consists of an extracellular domain (ECD), a transmembrane domain (TM), and an intracellular kinase domain (KD) (Figure 1*a*). Myc-His tagged ALK was constructed by inserting the complete ALK open reading frame (nucleotides 1-1620) into a commercially available mammalian expression vector, pcDNA3.1 *Myc-His* (Invitrogen). This construct was verified both by enzyme digestions and sequencing of the insertion sites (Data not shown). This Myc-His tag allowed the use of commercially available antibodies for immunoprecipitation, immunoblotting and the identification of ALK's immediate substrates.

The second part of this aim was to develop pull-down assay to show PTN binding to the ECD of ALK. To confirm that PTN binds to the ECD of ALK, ALK-ECD mutant construct

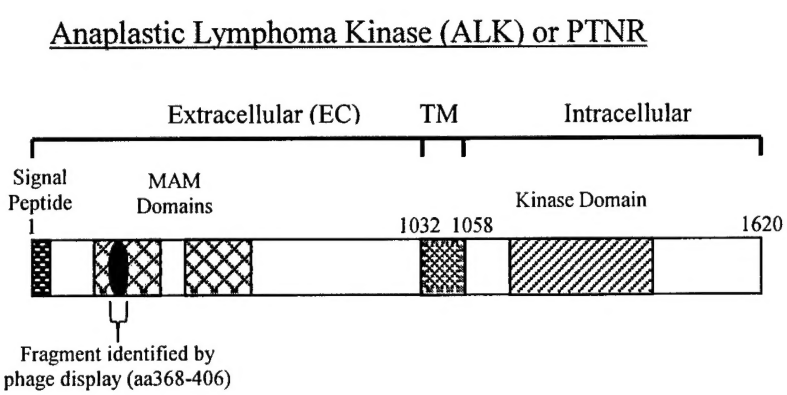
mentioned in last year's report was utilized (Figure 1*b*). Conditioned media from SW13 (human adrenal carcinoma) cells stably transfected with the empty vector (pcDNA) or with the ALK-ECD construct (ALK-ECD) were treated with purified PTN. Treated conditioned media were immunoprecipiated with an anti-PTN antibody and subsequently immunoblotted with an anti-Myc antibody (Figure 2). Conditioned media were used in these experiments because as mentioned in the previous reports, ALK-ECD is a secreted protein. Figure 2 shows that only the combination of conditioned media from SW13 ALK-ECD transfected cells and presence of PTN results in the interaction between PTN and ALK-ECD. This shows that PTN binds to the ECD domain of ALK.

The final task of Aim 3 was to mutant the ECD of ALK using full-length ALK as the template. The first part of this final task was to make gross deletions in the ECD of ALK using enzymatic digests. The first targeted deletion was the PTN/ALK interaction region originally found through phage display (3 and Figure 1*a*). Partial digests were employed to create the deletion due to lack of unique restriction digest sites surrounding the targeted region. When numerous attempts of partial digests failed to create an in-frame construct of ALK lacking amino acid 368-406 (ALKΔ368-406), site-directed mutagenesis method was used using Myc-His tagged ALK as the template. Due to the restriction of the method, only small portions (up to 10 bases) of the targeted region can be deleted each round. This method also proved to be difficult due to the size of the construct (~10.5 Kbp), although not impossible. While attempts were being made at generating ALKΔ368-406, other ALK mutants were also being made using the same the site-directed mutagenesis method. Although the generation of ALKΔ368-406 is still ongoing, the method proved to be working because ALK mutant constructs (mutated IRS-1-

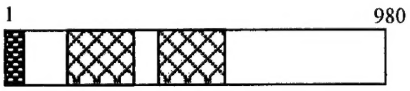
binding site, mutated SHC-binding site and mutated lysine in the ATP substrate-binding motif) were successfully made.

FIGURE 1

A.



B.



ALK-ECD

FIGURE 1: Construction of ALK dominant-negative mutants. *A.* A diagram illustrating the domains of ALK. *B.* A schematic of the illustrating the portions of ALK inserted into a mammalian expression vector. ALK-ECD represents 1-980 amino acids.

FIGURE 2

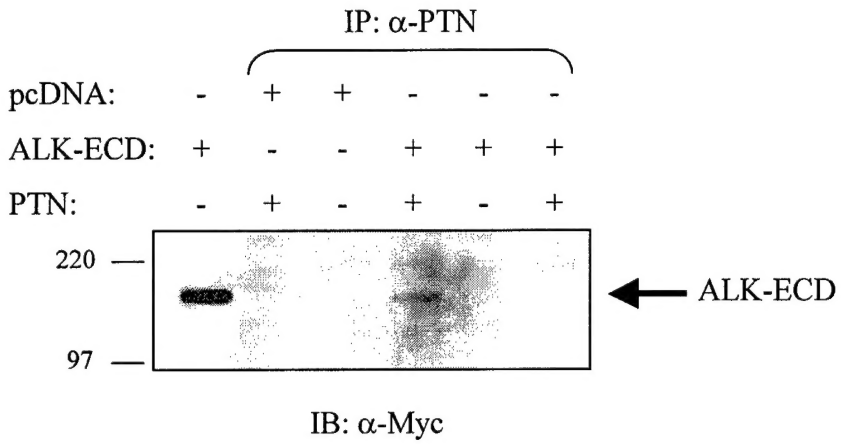


Figure 2: Interaction of PTN and ECD of ALK in SW13 cells. Conditioned media from SW13 cells stably transfected with either pcDNA or ALK-ECD were treated with or without PTN and subjected to immunoprecipitation with an anti-hPTN antibody for 1 hour. Samples were subsequently immunoblotted with an anti-Myc antibody to detect Myc-His tagged ALK-ECD.

KEY RESEARCH ACCOMPLISHMENTS

- Tagged full-length ALK was constructed by insertion of ALK coding region into an INVITROGEN mammalian expression vector containing a Myc/His tag (Figure 1).
- Interaction between PTN and ALK was determined by immunoprecipitation with an anti-PTN antibody followed by western blot, using an anti-myc antibody (Figure 2).
- Site-directed mutagenesis method was used to create ALK mutant constructs.

REPORTABLE OUTCOMES

Doctoral Dissertation:

"The Role of Insulin Receptor Substrate-1 in Anaplastic Lymphoma Kinase Signaling," April 2003.

Abstracts:

Kuo AH, Stoica GE, Riegel AT, Wellstein A. (2002) *Abrogation of IL-3 dependence requires co-expression of the anaplastic lymphoma kinase receptor and its major substrate, IRS-1, in 32D cells.* 93rd Annual Meeting, American Association for Cancer Research, San Francisco, CA.

Kuo AH, Stoica GE, Riegel AT, Wellstein A. (2002) *Insulin receptor substrate 1 is a rate-limiting factor in anaplastic lymphoma kinase signaling.* Era of Hope 2002 Department of Defense Breast Cancer Research Program Meeting, Orlando, Fl.

CONCLUSIONS

While the above-described experiments did not yield all positive results (i.e. ALK mutant lacking region identified by phage display), they did meet the goals described in Task 3 of the approved grant application. The construction of tagged ALK facilitated in identification of several immediate substrates of activated ALK (data not shown). Due to the lack of a good commercially available ALK antibody, this construct is an invaluable tool in the investigation of ALK's downstream signaling which is currently ongoing. Results from the second part of Task 3 showed that PTN does interact with ALK. Only when the SW13/ALK-ECD conditioned media was mixed with PTN, can ALK-ECD be co-immunoprecipitated with PTN (Figure 2). This result confirmed what was observed previously with phage display (3). Although the construct of ALKΔ368-406 has not yet been obtained, the generations of other ALK mutants prove that the site-directed mutagenesis method can be employed to create ALK mutants. Further attempts at generating ALKΔ368-406 are currently underway using a new commercially available site-directed mutagenesis kit for large plasmids (from Stratagene).

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